Pergamon

0022-1910(94)00132-4

J. Insect Physiol. Vol. 41, No. 6, pp. 489–500, 1995
Elsevier Science Ltd. Printed in Great Britain

Influence of Development and Prothoracicotropic Hormone on the Ecdysteroids Produced *In Vitro* by the Prothoracic Glands of Female Gypsy Moth (*Lymantria dispar*) Pupae and Pharate Adults

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Received 23 February 1994; revised 25 October 1994

Fluctuations in hemolymph ecdysteroid titer are part of a complex mechanism that regulates pupal–adult development. The amount of ecdysteroid produced *in vitro* by prothoracic glands from female *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) pupae and pharate adults, as well as the competency of these glands to respond to a prothoracicotropic hormone (PTTH) stimulus *in vitro*, each correspond temporally with hemolymph ecdysteroid titers. Based on studies of gland kinetics and dose–responses to brain extract using prothoracic glands from different female pupal and pharate adult ages, an *in vitro* bioassay for the quantification of PTTH activity was developed using glands from day 2 females incubated without stimulus for 1 h followed by a 3 h incubation with stimulus. Only extracts of brains and corpora allata from pupae and pharate adults possess a PTTH factor. This factor is heat stable and can be separated on high performance size exclusion chromatography into two molecular sizes of 13.75 and 3.2 kDa. Ecdysone and 3-dehydroecdysone are produced *in vitro* by prothoracic glands from all ages of female *L. dispar* pupae and pharate adults tested. The amount of ecdysone produced by these glands exceeds that of 3-dehydroecdysone production after 4 h of incubation.

Neuropeptide hormone Pupae Pharate adult Feedback regulation Juvenile hormone Ecdysteroid synthesis and release

INTRODUCTION

Fluctuations in hemolymph ecdysteroid titer are part of a complex mechanism that regulates larval–pupal development in Lepidoptera (Bollenbacher, 1988). These fluctuations correlate temporally with fluctuations in the *in vitro* production of ecdysone by prothoracic glands from last instars (Bollenbacher *et al.*, 1975; Okuda *et al.*, 1985; Ciancio *et al.*, 1986; Kelly *et al.*, 1992). Differences do exist between the amount of ecdysone produced by larval glands *in vitro* and the capacity of these glands to be influenced by different regulators such as prothoracicotropic hormone (PTTH) (Sakurai *et al.*, 1989a; Ciancio *et al.*, 1986; Watson and Bollenbacher, 1988; Watson *et al.*, 1987, 1988). For example, in last instar gypsy moths, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), the responsiveness of glands *in vitro* to brain extracts containing PTTH peaks on day 5, however, both the production of ecdysone by prothoracic glands *in vitro* and the hemolymph ecdysteroid titer peak on day 10 (Kelly *et al.*, 1986, 1992).

As in larval–pupal development, fluctuations in hemolymph ecdysteroid titer during pupal–adult development are part of a complex regulatory mechanism. This regulation results in adult cuticle formation (Riddiford, 1985), eclosion (Truman, 1985) and oogenesis (Hagedorn and Kunkel, 1979; Hagedorn, 1985; Postlethwait and Giorgi, 1985; Tsuchida *et al.*, 1987). Prothoracic glands from Lepidoptera pupae increase their ecdysteroid production *in vitro* in response to PTTH (Bollenbacher *et al.*, 1979; Agui *et al.*, 1983; Nagasawa *et al.*, 1984a). However, the exact nature of the function of prothoracic glands and PTTH in regulating hemolymph ecdysteroid levels during pupal–adult development is not known in Lepidoptera.

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PTTH appears to exist in at least two molecular weight forms in the adult brains of *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Ishizaki *et al.*, 1983; Nagasawa *et al.*, 1984a), the larval brains of *L. dispar* (Masler *et al.*, 1986; Kelly *et al.*, 1991,Kelly *et al.*, 1992) and *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Bollenbacher *et al.*, 1984) and the pupal brains of *M. sexta* (O'Brien *et al.*, 1986). Each molecular weight form of PTTH stimulates a dose-dependent increase in the *in vitro* production of ecdysteroids by prothoracic glands from the pupal stage of *M. sexta* (O'Brien *et al.*, 1986).

Temporal relationships among the amount of ecdysteroid produced *in vitro* by prothoracic glands, gland responsiveness to PTTH and hemolymph ecdysteroid titer, are not as well understood in Lepidoptera pupae and pharate adults as they are in larvae. A detailed study of the neuroendocrine regulation of the ecdysteroid milieu has not been reported for pupal–adult development of *L. dispar*. The major goal of the current study is to determine these temporal relationships in pupae and pharate adults of female *L. dispar* by investigating the influence of PTTH on the kinetics of ecdysteroid production by prothoracic glands *in vitro*. An *in vitro* bioassay for PTTH was devised using developmental and kinetic information on ecdysteroid production by glands from female *L. dispar* pupae and pharate adults. A preliminary investigation on development of this assay was previously published (Fescemyer *et al.*, 1990). We report here the complete development of this bioassay and its use in characterizing the sources and forms of PTTH in females, fluctuations in the capacity of pupal and pharate adult prothoracic glands to respond to PTTH and changes in hemolymph ecdysteroid titers.

Ecdysone was the only ecdysteroid known to be secreted by prothoracic glands *in vitro* until Warren *et al.* (1988) observed that 3-dehydroecdysone is the primary ecdysteroid secreted by glands from *M. sexta* larvae and pupae. This 3-dehydroecdysone is rapidly converted to ecdysone by the hemolymph enzyme 3-oxoecdysteroid 3β-reductase (Sakurai *et al.*, 1989b; Kiriishi *et al.*, 1990). Glands from different ages of *L. dispar* pupae and pharate adults were incubated *in vitro* to determine which and how much of these ecdysteroids are produced. We report that 3-dehydroecdysone is produced *in vitro* by glands from gypsy moth pupae and pharate adults, but at amounts similar to or lower than ecdysone.

MATERIALS AND METHODS

Insect rearing

The source and rearing of *L. dispar* have been reported previously (Masler *et al.*, 1991). Larvae were reared through the fourth stadium at a density of 10/180 ml cup. Sexes were segregated by size during the fourth instar, when females are clearly larger than males. Last (fifth) larval instars were reared at a density of 5/180 ml cup. Females were used in all experiments. Pupae were selected 5 h after lights-on, following larval–pupal ecdysis during the previous scotophase. These pupae were designated as day 1. The exact time for apolysis of the pupal cuticle was not determined. Pharate adult features, such as antennae, optic lobes of the brain, etc., begin to develop in day 3 females. The fat body of day 3 females appears granular-like instead of lobe-like and has begun to breakup and undergo histolysis. Pharate adult development probably begins 2–3 days after the larval–pupal molt. This investigation covers both pupal and pharate adult development.

Organ dissection and extraction procedures for PTTH

Insects were anesthetized with CO_2. Dissection of all tissue (brains, corpora allata, corpora cardiaca and prothoracic glands) was performed while the opened body cavity was bathed in *Bombyx* saline (Okuda *et al.*, 1985). Adhering fat body, muscle and tracheae were carefully removed. Dissected tissues were washed for 5 min in saline before they were used except for prothoracic glands, which were taken through an additional 5 min wash in fresh saline to reduce hemolymph contamination.

Brains, pairs of corpora allata or pairs of corpora cardiaca were transferred from the saline wash to individual polypropylene microhomogenizers (1.5 ml conical tubes with matching pestles; Kontes, Vineland, NJ) on dry ice. At the end of a dissection period, homogenizers with tissue were stored at −80°C. Frozen tissue was thawed on ice and homogenized at 4°C in Grace's medium (GIBCO, Grand Island, NY) at a concentration of 3 tissue equivalents/25 μl of medium (a tissue equivalent = one brain, one pair of corpora allata or one pair of corpora cardiaca). Immediately after homogenization, tissue extracts were either heated for 2 min in a 100°C water bath and then centrifuged (12,000 *g*, 4°C, 10 min; "boiled extract") or centrifuged only ("crude extract").

Doses (0.001–3 brain, corpora allata pair or corpora cardiaca pair equivalents/25 μl) of the boiled or crude extracts were prepared by dilution with Grace's medium. One aliquot of each dose was tested for PTTH activity the day the extract was prepared and the rest were stored at −20°C and tested the next day. In experiments testing the influence of PTTH, incubation time and age on ecdysteroid production by prothoracic glands *in vitro* (Fig. 2), only one pupal or pharate adult age could be tested per day. Therefore, glands from a particular age, which was selected randomly, were stimulated with an aliquot (1/age) of the same brain extract stored at −20°C.

Incubation of prothoracic glands, assay for PTTH and time–course for ecdysteroid production in vitro

Prothoracic glands were incubated in 25 μl standing drops of medium placed in a covered, polystyrene culture dish (9 × 50 mm). Incubations were conducted in a humidified chamber, 26°C, constant darkness, with either Grace's medium, boiled or crude tissue extracts or high

pressure liquid chromatography (HPLC) fractions prepared in Grace's medium.

In the standard assay for PTTH activity, the gland from the right side of an animal served as the control and the contralateral gland was the experimental gland. At the end of the first hour of incubation, the medium of the control gland was replaced with fresh medium while the medium of the experimental gland was replaced with medium containing test extract or HPLC fraction. Glands were then incubated for another 3 h, and medium was collected for radioimmunoassay (Borst and O'Connor, 1972; see below).

The time–course for ecdysteroid production *in vitro* was determined by incubating glands from different pupal and pharate adult ages for up to 9 h and using three medium replacement protocols. Time zero values in all medium replacement protocols were determined with ecdysteroid extracted from glands that were not incubated. In the first protocol, the same set of glands was used for each time point, but the medium was removed for radioimmunoassay and replaced with an equal volume of fresh medium. This protocol was used to examine the influence of medium replacement on the time–course of ecdysteroid production. In the second protocol, a different set of glands was used for each incubation time tested (1, 2, 4, 6 or 9 h). Medium was removed for radioimmunoassay at the end of each incubation period. This protocol was used to examine the influence of no medium replacement on the time–course of ecdysteroid production in order to determine if ecdysteroid released into the media regulates ecdysteroid production by a feedback mechanism. In the third protocol, the incubation medium of sets of gland pairs from individual animals was replaced as described in the first protocol except that, beginning at 2 h and for all subsequent times, the medium of the experimental gland was replaced with boiled extract of day 1 pupal brains (0.25 equivalent/25 μl). This protocol examined the influence of a brain extract stimulus on the time–course of ecdysteroid production.

Radioimmunoassay

The ecdysteroid radioimmunoassay has been previously described (Kelly *et al.*, 1986; Masler and Adams, 1986). The antibody was a gift from W. E. Bollenbacher (University of North Carolina, Chapel Hill) and has been characterized (Gilbert *et al.*, 1977). Ecdysone (Sigma Chemical Co.) was used as the unlabeled ligand competing with [23,24-^{3}H(N)]ecdysone (60–80 Ci/mmol; NEN Research Products, Boston, MA).

Characterization of the molecular weight of PTTH

Brains, corpora allata and corpora cardiaca were dissected from the same day 2 females and washed as described above. Tissues were homogenized in methanol:water:trifluoroacetic acid (90:10:0.1, v/v/v) and centrifuged (12,000 ***g***, 4°C, 10 min). The volume of the resulting supernatant was reduced, but not dried, in a vacuum concentrator and fractionated on an HPLC pumping (1 ml/min) acetonitrile:water:trifluoroacetic acid (40:60:0.1, v/v/v) through two Protein-Pak 125 (7.8 mm × 30 cm, Waters Chromatography Division, Millipore Corp., Milford, MA) size exclusion columns linked in tandem. Fractions were collected every 0.5 min. The fractions were dried in a centrifugal vacuum concentrator, dissolved in Grace's medium and stored at 4°C until assayed. Molecular weight markers were carbonic anhydrase (29 kDa) and cytochrome *c* (12 kDa) from Sigma Chemical Co. (St Louis, MO), and whole myoglobin (16.95 kDa) myoglobin I + II (14.4 kDa), myoglobin I (8.16 kDa), myoglobin II (6.214 kDa) and myoglobin III (2.512 kDa) from LKB-Producter AB (Bromma, Sweden).

Ecdysteroid analyses

Media from prothoracic gland incubations were collected into tubes containing methanol (methanol:medium, 75:25, v/v; 4°C). Prothoracic glands were homogenized in 50 μl/gland of methanol:water (75:25, v/v; 4°C). Precipitation of insoluble material in the methanolic extracts was continued for at least 24 h at −20°C. Particulate matter and precipitate were removed by centrifugation (12,000 ***g***, 4°C, 10 min). The resulting supernatant was collected as the "methanol extract" and stored at −20°C. Aliquots of the extracts were dried in a centrifugal vacuum concentrator, and ecdysteroid levels determined by radioimmunoassay (see above). Only background levels (±5 pg/25 μl of medium) of radioimmunoassay activity were observed in the methanol extracts of the different media (Grace's medium, HPLC fractions or the various doses of boiled and crude extracts) used in the prothoracic gland incubations.

Characterization of the ecdysteroids synthesized in vitro

Prothoracic glands from day 1, 2, 3, 4, 5 and 7 female pupae and pharate adults were dissected, washed twice in saline and incubated in Grace's medium using the second protocol (see above). At the end of the incubation time tested (1, 4 or 9 h), the media from three pairs of glands were combined and extracted with methanol (see above). The methanol extract was dried in a centrifugal vacuum concentrator, dissolved in 25 μl of 4°C methanol:water (41:59, v/v) and stored at −20°C for no more than 3 days before HPLC analysis was performed. The extracts were fractionated on a Hewlett–Packard 1090A liquid chromatograph (Wilmington, DE). Mobile phase was 41% aqueous methanol, flow rate was 1 ml/min and the reverse-phase column used was a Supelcosil LC-18-DB, 5 cm × 4.6 mm i.d. (Supelco Inc., Bellefonte, PA). Peaks were detected at 243 nm. Known quantities of ecdysone, 20-hydoxyecdysone, makisterone A (Sigma Chemical Co.) and 3-dehydroecdysone (a gift from M. Thompson, Insect Neurobiology and Hormone Laboratory, Beltsville, MD) were used as external standards to quantify the integrated peak areas and determine the

precision (>95%) of the extraction and HPLC method. All ecdysteroid standards were purified by reverse-phase HPLC before use.

Unknown peaks with retention times similar to standard ecdysone and 3-dehydroecdysone were collected, purified by a second reverse-phase HPLC and analyzed by methane chemical ionization mass spectrometry. Spectra were obtained from a Finnigan 4510 mass spectrometer equipped with an Incos data system. The instrument was calibrated by analysis of perfluorotributylamine. Ionization conditions were methane at 0.3 torr and a source temperature of 100°C. Samples were deposited as methanolic solutions onto the desorption probe loop. The methanol was removed by an air stream before the probe was inserted into the instrument and heated by the application of an electric current increasing at 20 ma/s. Spectra were obtained from m/z 90–570 with a scan time of 0.85 s.

Data analysis

Dose–responses for the activation of prothoracic glands by extracts were determined using the amount of ecdysteroid produced or the activation ratio as the dependent (Y) variable. The activation ratio is defined as the amount of ecdysteroid detected in medium following incubation of an experimental gland divided by the amount detected in medium containing a control gland taken from the same larva. The final activation ratio for each concentration of tissue extract assayed was the mean of the activation ratios obtained from 5 or more gland pair assays. The effective dose needed to achieve half-maximal ecdysteroid production or activation (ED_{50}) was used to compare responsiveness of different glands to the same source of PTTH or responsiveness of the same glands to different sources of PTTH. ED_{50} values were calculated using the PROBIT procedure of PC-SAS (SAS Institute, 1988) and degrees of freedom (d.f.) and χ^2 values are for the Pearson goodness-of-fit tests.

Factorial designs were used to test the influence of age and incubation time [7 × 10 × 2 for Fig. 1(A); 7 × 16 × 2 for Fig. 1(B)], the influence of age, incubation time and gland treatment (6 × 5 × 2 for Fig. 2) and the influence of age, incubation time and type of ecdysteroid (6 × 3 × 2 for Fig. 8) on the amount of ecdysteroid produced. Regression analysis was used to test the linear relationship between amount of ecdysteroid produced and incubation time [Figs 1(A), (B) and 2]. A factorial design was used to test the influence of boiling a brain extract and dose [2 × 7 for Fig. 3(A) and(B)], and type of tissue extract (brains or corpora allata) and dose (2 × 7 for Fig. 5) on the amount of ecdysteroid produced and activation ratio. Probability (P) values in the results section are for the independent effects type III sums of squares F-test. Student's t-test (Steel and Torrie, 1980) was used to examine differences in the mean amounts of ecdysteroid compounds (H_0: $\mu_1 = \mu_2$) synthesized during a particular period of incubation. This test was also used to determine if means were different from zero (H_0: $\mu = 0$). The relationship between amounts (ng) of ecdysteroid and activation ratio were tested using Pearson product–moment correlations (Steel and Torrie, 1980). Values in the results section are means ± SE.

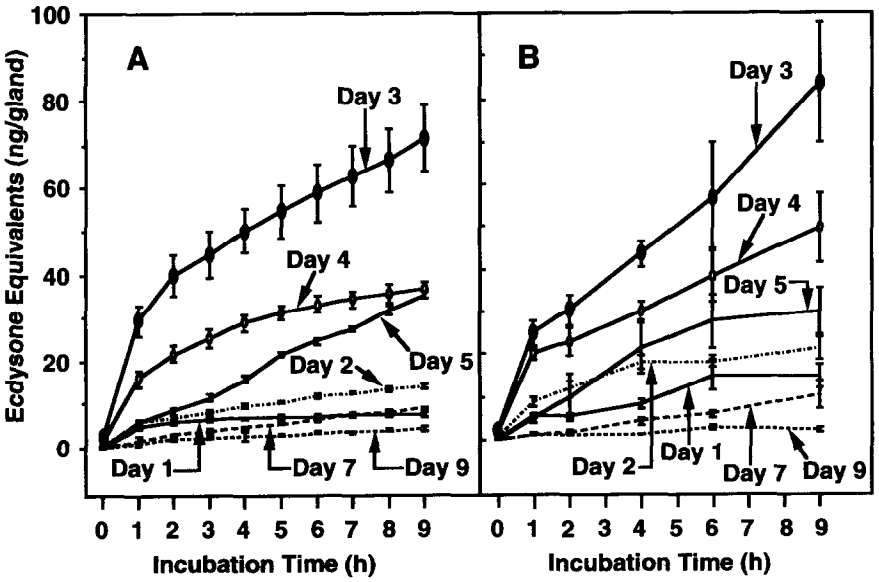

FIGURE 1. Influence of incubation time and medium replacement on the *in vitro* production of ecdysteroid by prothoracic glands from different ages of female *L. dispar* pupae and pharate adults. Points represent means ± SE (n = 5–6). Time zero represents the ecdysteroid extracted from glands that were not incubated. Eclosion occurred on day 10. (A) Cumulative ecdysteroid production in response to medium (Grace's) replacement where the same gland within an age was used for each time point by removing the medium (25 μl) for radioimmunoassay and replacing it with fresh medium (25 μl). The amount of ecdysteroid produced by each gland tested is the sum of the amount of ecdysteroid produced at that time and all earlier times. (B) Ecdysteroid produced without medium replacement where a different gland was used for each time tested and the medium was removed for radioimmunoassay at the end of the incubation time being tested.

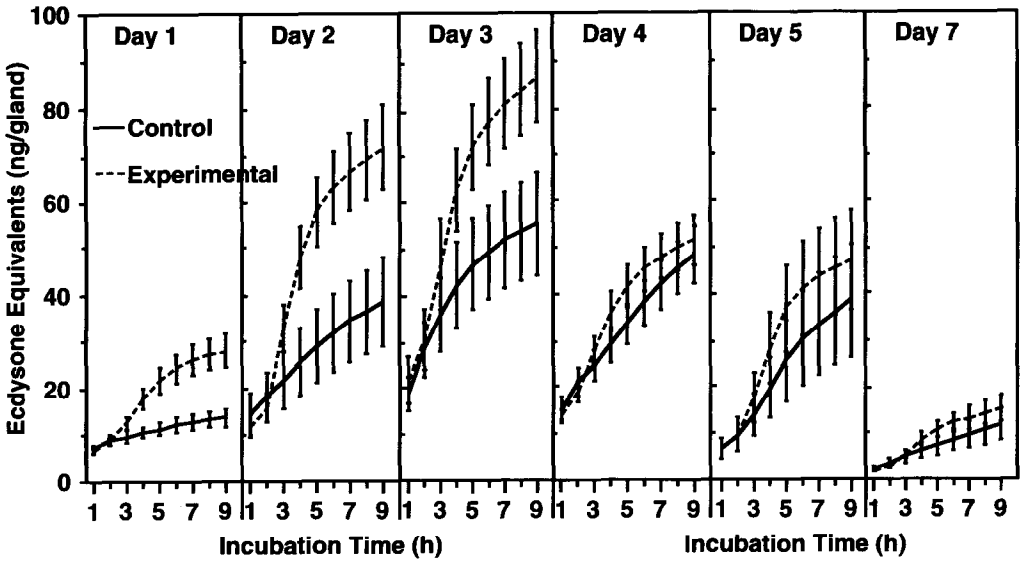

FIGURE 2. Influence of PTTH stimulation and incubation time on cumulative ecdysteroid produced *in vitro* by prothoracic glands from different ages of female *L. dispar* pupae and pharate adults. Eclosion occurred on day 10. Points represent means ± SE (n = 5–6 pairs of glands). The same pair of glands from an individual was used for each time point by removing the Grace's medium (25 μl) for radioimmunoassay and replacing it with fresh medium (25 μl). Beginning at 2 h and continuing for all further time points, the medium of the experimental gland was replaced with boiled extract of day 1 female pupal brains (0.25 equivalent/25 μl). Amount of ecdysteroid produced by each gland tested is the sum of the amount of ecdysteroid produced at that time and all earlier times. Brains were extracted with 4°C Grace's medium.

PC-SAS was used to perform all statistical analyses and graphs (SAS Institute, 1988).

RESULTS

Time–course of ecdysteroid production

Ecdysteroid production by prothoracic glands increased rapidly during the first 1–2 h of incubation in Grace's medium followed by a slow, constant increase in production through 9 h [Fig. 1(A) and (B)]. This pattern was similar for glands from all ages of pupae and pharate adults tested and for each of the medium replacement protocols tested. Coefficients of simple determination (r^2) for linear correlations ($P \leq 0.031$, $n = 50$) between ecdysteroid production and incubation times 0–9 h were 0.55, 0.57, 0.31, 0.37, 0.72, 0.82, 0.85 for ages 1, 2, 3, 4, 5, 7 and 9, respectively. Linear correlations ($P \leq 0.001$, $n = 40$) between ecdysteroid production and incubation times 1–9 h had the highest r^2 values or 0.87, 0.88, 0.91, 0.84, 0.82, 0.89, 0.87 for ages 1, 2, 3, 4, 5, 7 and 9, respectively.

The amount of ecdysteroid produced was highest in control glands from day 3 females and lowest in glands from day 9 females [Fig. 1(A) and (B)]. Eclosion occurred on day 10. The amount of ecdysteroid in time zero glands did not vary ($P \geq 0.6$) with age and was not different from 0 ($P > |t| \geq 0.7$). During incubation times greater than 0, differences ($P \leq 0.001$) between the ecdysteroid produced by glands from the pupal and pharate adult ages tested are day $3 > 4 > 5 > 2 \geq 1 \geq 7 \geq 9$. Similar patterns of ecdysteroid production variation, relative to incubation time and age, were observed with each medium replacement protocol tested [Fig. 1(A) and (B)].

Each prothoracic gland from an individual produced ecdysteroid in an amount similar ($P \geq 0.8$) to its partner prior to stimulation of the experimental gland at 2 h with a boiled extract of brains from day 1 females (Fig. 2). The control gland continued to produce ecdysteroid in constant amounts for incubation times 1–9 h ($P \leq 0.001$, $n = 63$; r^2 was 0.96, 0.94, 0.88, 0.85, 0.87 and 0.87 for ages 1, 2, 3, 4, 5 and 7, respectively). The experimental gland increased its ecdysteroid production for 2–3 h after the addition of brain extract (Fig. 2). This increase is followed 3 h after addition of stimulus by a constant increase in ecdysteroid production which parallels the amount produced by the control gland (incubation times 5–9 h in Fig. 2; $P \leq 0.001$, $n = 63$, r^2 when incubation times 5–9 h were used was 0.87, 0.89, 0.9, 0.81, 0.88 and 0.83 for ages 1, 2, 3, 4, 5 and 7, respectively). Cumulative amounts of ecdysteroid produced during incubation times 4–9 h by experimental glands from days 1 to 3 were greater than ($P \leq 0.015$) that produced by control glands (Fig. 2). Although cumulative amounts of ecdysteroid produced during incubation times 3–9 h by experimental glands from days 4 to 7 were greater than that produced by control glands, these differences were not significant ($P \geq 0.17$). The greatest difference in ecdysteroid production between control and experimental glands was observed for those from day 2 females followed by day $3 > 1 \geq 5 \geq 4 \geq 7$ (Fig. 2).

Using the third medium replacement protocol, different combinations of pre-stimulation periods (1 and 2 h), in which the experimental gland was incubated without stimulation, followed by different post-stimulation

periods (2 and 3 h) with a boiled extract of brains (0.25 equivalent/25 μl) from day 2 females were tested using glands ($n = 5$ per combination) from day 2 females. The pre-stimulation:post-stimulation incubation period combination of 1:3 h resulted in the highest activation ratio (3.36 ± 0.07) followed by 1:2 (2.91 ± 0.07) > 2:2 (2.19 ± 0.08) > 2:3 (2.12 ± 0.06).

Activation of ecdysteroid production in prothoracic glands by different sources of PTTH

Concentrations of crude or boiled extracts of day 2 female brains above 0.001 brain equivalent/25 μl stimulated a dose-dependent increase ($P \leq 0.001$) in the amount of ecdysteroid produced [Fig. 3(A)]. The ED_{50} values for activation were similar for the crude (0.12 brain equivalent/25 μl) and boiled extracts ($ED_{50} = 0.1$ brain equivalent/25 μl). No differences were observed between crude and boiled extracts when the dose–response was determined using amount of ecdysteroid produced [Fig. 3(A); $P \geq 0.17$] or activation ratio [Fig. 3(B); $P \geq 0.22$]. The amount of ecdysteroid produced by the experimental glands [Fig. 3(A)] is correlated ($P > |r| \leq 0.001$, $r = 0.92$, $n = 35$) with the activation ratio [Fig. 3(B)]. This correlation indicates that activation ratio can be used in place of the amount of ecdysteroid produced because these data sets correspond to each other. Therefore, only the activation ratio dose–responses are reported for all further experiments.

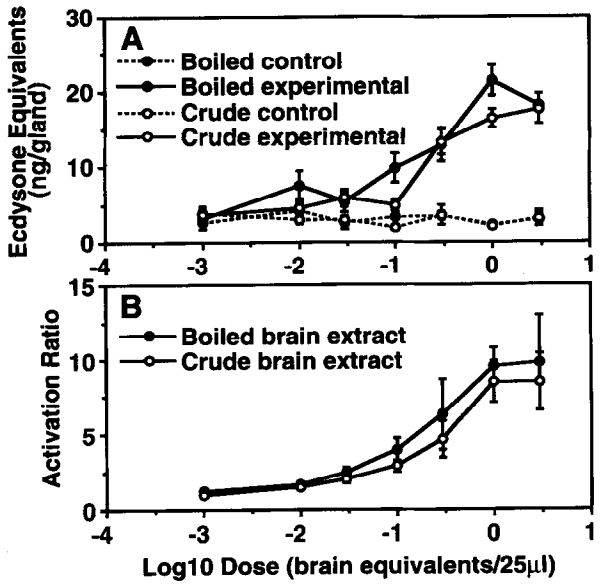

FIGURE 3. Influence of a boiled or crude extract of brains on the amount (A) and activation (B) dose-dependent responses of ecdysteroid production by the prothoracic glands *in vitro*. Points represent means ± SE ($n = 5$–6 pairs of glands). Brains and gland pairs were dissected from day 2 female pupae of *L. dispar*. Brains were extracted with 4°C Grace's medium. One aliquot of the extract was immediately boiled for 2 min and centrifuged (12,000 *g*, 4°C, 10 min). The supernatant (12,000 *g*, 4°C, 10 min) of the remaining extract was used as the "crude extract." One gland of a pair of glands from an individual served as the control, which received no extract, while the contralateral gland was incubated without stimulation for 1 h, followed by replacement of its medium with extract and a 3 h incubation with extract. The amount of ecdysteroid produced by control glands did not exceed 4 ng.

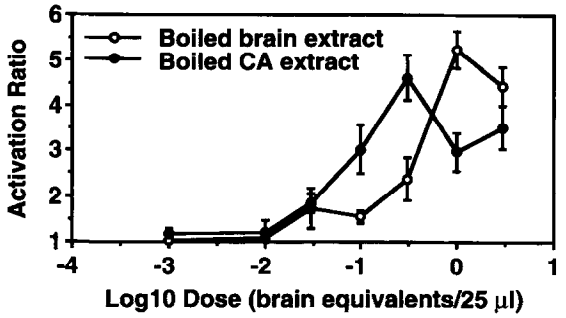

FIGURE 4. Influence of boiled extracts of brains (open symbols) and corpora allata (CA; solid symbols) on the activation of ecdysteroid production by the prothoracic glands *in vitro*. Points represent means ± SE ($n = 5$–6 pairs of glands). Brains, corpora allata and glands were dissected from day 2 female pupae or pharate adults of *L. dispar*. See Fig. 3 for methods used in extract preparation and assay for PTTH activity.

Differences ($P \leq 0.009$) between boiled extracts of brains and corpora allata from day 2 females were observed in their ability to activate ecdysteroid production by glands from day 2 females (Fig. 4). Activation ratio dose–responses yielded ED_{50} values of 0.24 equivalent/25 μl for brains and 0.04 equivalent/25 μl for corpora allata (Fig. 4). Similar results (data not shown) were obtained using tissues from day 1 females. Boiled extracts of corpora cardiaca did not activate ecdysteroid production even at 3 corpora cardiaca pair equivalents/25 μl (data not shown).

Glands from day 1 females were stimulated with different concentrations of boiled extracts of brains from day 1 or 2 females (Fig. 5). The ED_{50} for the activation ratio dose–response to day 1 brains (0.095 equivalent/25 μl) was greater than that for day 2 brains (0.07 equivalent/25 μl), but maximum activation occurred at the same dose or 1 brain equivalent/25 μl (Fig. 5). Day 1 brains stimulated more ecdysteroid production at doses of 0.3–3 equivalents/25 μl ($P \leq 0.02$) than did day 2 brains.

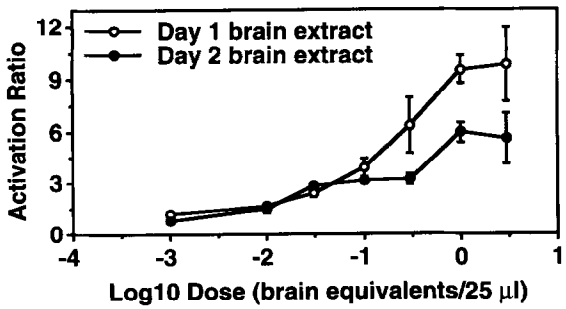

FIGURE 5. Influence of boiled extracts of brains from day 1 (open symbols) and day 2 (solid symbols) pupae or pharate adults on the activation of ecdysteroid production by day 2 pupal or pharate adult prothoracic glands *in vitro*. Points represent means ± SE ($n = 5$–6 pairs of glands). Brains and glands were dissected from day 1 female pupae of *L. dispar*. See Fig. 3 for methods used in extract preparation and assay for PTTH activity.

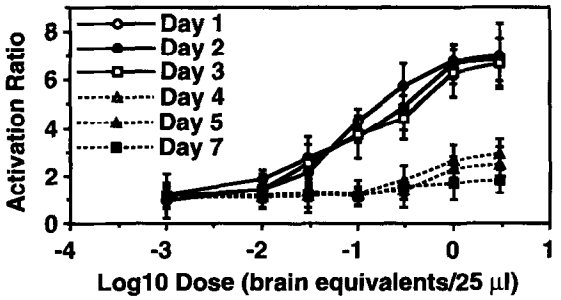

FIGURE 6. Activation of ecdysteroid production by prothoracic glands from different ages of female *L. dispar* pupae and pharate adults. All glands were stimulated by doses of the same boiled extract of brains from day 2 female pupae or pharate adults of *L. dispar*. Points represent means ± SE (n = 5 pairs of glands). See Fig. 3 for methods used in extract preparation and assay for PTTH activity.

Responsiveness of prothoracic glands from different pupal and pharate adult ages

Boiled extract of brains from day 2 females activated ecdysteroid production in a dose-dependent manner in glands from all female ages tested (Fig. 6). The dose–responses of glands from days 1–3 are different ($P \leq 0.001$) from those of glands from days 4, 5 and 7. Threshold dose–responses, ED_{50} values and the maximum activation ratios varied between these two age groups. Glands from days 1–3 respond to a threshold dose (about 0.01 equivalent/25 µl) that is about 30-fold lower than the threshold dose (about 0.3 equivalent/25 µl) for glands from days 4, 5 and 7. Values for the ED_{50} are lowest for day 2 (0.159 equivalents/25 µl; d.f. = 4, $\chi^2 = 1.43$) followed by day 1 (0.210 equivalents/25 µl; d.f. = 4, $\chi^2 = 4.46$) > day 3 (0.264 equivalents/25 µl; d.f. = 4, $\chi^2 = 1.20$) > day 4 (0.522 equivalents/25 µl; d.f. = 4, $\chi^2 = 1.16$) > day 5 (0.770 equivalents/ 25 µl; d.f. = 4, $\chi^2 = 1.49$) > day 7 (0.927 equivalents/25 µl; d.f. = 4, $\chi^2 = 1.86$). The maximum activation ratios for glands from all ages occurred at a dose of 3 equivalents/25 µl (Fig. 6). Maximum activation ratios (6.87 ± 1.08) for glands from days 1–3 females are about 2.8-fold greater than the maximum activation ratios for glands from days 4, 5 and 7 (2.42 ± 0.87).

Characterization of the molecular weight of PTTH

Brains and corpora allata were dissected from day 2 females. Extracts of both tissues produced two peaks of PTTH activity when they were fractionated on a size exclusion system [Fig. 7(A) and (B)]. Molecular weights were estimated to be 3.12 and 13.75 kDa by using the linear regression equation ($r^2 = -0.98$), $y = 0.245x - 7.04$, where y is $\log_{10}$ molecular weight and x is retention time in minutes.

Characterization of the ecdysteroids produced in vitro

Reverse-phase HPLC separated two peaks from the methanol extracts of media from all incubation times tested with control prothoracic glands from all female pupal and pharate adult ages tested (Fig. 8). These peaks had u.v. light absorbance maxima at 243 nm. Retention times for these peaks were the same as those for pure standards of ecdysone (6.87 min) and 3-dehydroecdysone (7.84 min), while 20-hydroxyecdysone and makisterone A eluted at 3.31 and 5.69 min, respectively. The material that co-eluted with ecdysone had a mass spectrum that was identical to ecdysone. Methane chemical–ionization mass spectrometry of the putative ecdysone (about 550 ng) produced the following ions (m/z, relative intensity), 505 (M + C_3H_5, 9%), 493 (M + C_2H_5, 23%), 465 (M + H, 45%), 447 (M + H − H_2O, 72%), 429 (M + H − $2H_2O$, 100%), 411 (M + H − $3H_2O$, 35%), 348 (M + H − 117 for fission of the side chain at C_{20}–C_{22}, 5%), 331 (348 − H_2O, 9%), 313 (348 − $2H_2O$, 5%) and 99 (C_{22}–C_{27} side chain less H_2O, 30%). The unknown peak with a retention time similar to standard 3-dehydroecdysone could not be confirmed by mass spectrometry because of contamination and the small amount obtained (about 70 ng).

In vitro production of ecdysone and 3-dehydroecdysone by prothoracic glands from different female pupal and pharate adults ages

Glands from all ages tested produced similar ($P \geq 0.18$) amounts of ecdysone and 3-dehydroecdysone as

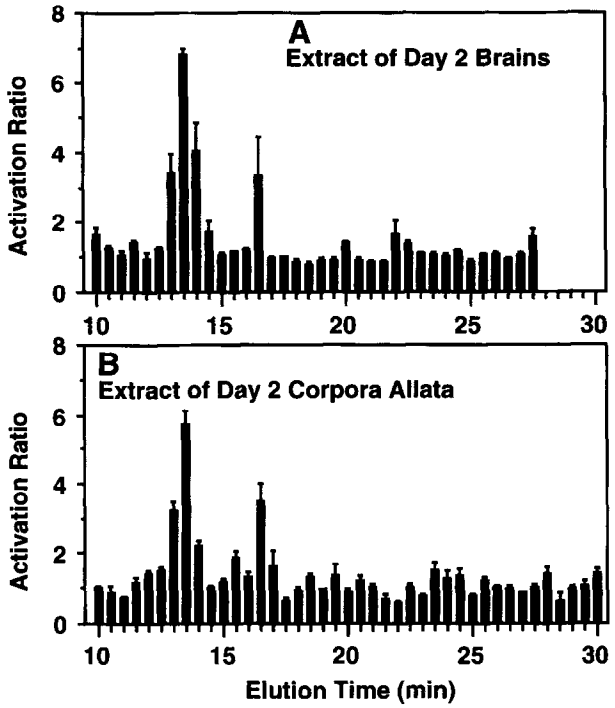

FIGURE 7. Fractionations of brain (A) and corpora allata (B) extracts by HP–SEC. Bars represent means ± SE (n = 3 pairs of prothoracic glands). Brains, corpora allata and glands were dissected from day 2 female pupae or pharate adults of *L. dispar*. Brains and corpora allata were extracted with methanol:water:trifluoroacetic acid (90:10:0.1, v/v/v) and centrifuged (12,000 *g*, 4°C, 10 min). The volume of the resulting supernatant was reduced, but not dried, in a vacuum concentrator and fractionated on an HP–SEC system pumping (1 ml/min) acetonitrile:water:trifluoroacetic acid (40:60:0.1, v/v/v) through two Protein-Pak 125 (7.8 mm x 30 cm, Waters Chromatography Division, Millipore Corp., Milford, MA) size exclusion columns linked in tandem. Fractions were dried, dissolved in Grace's medium and assayed for PTTH activity as described in Fig. 3.

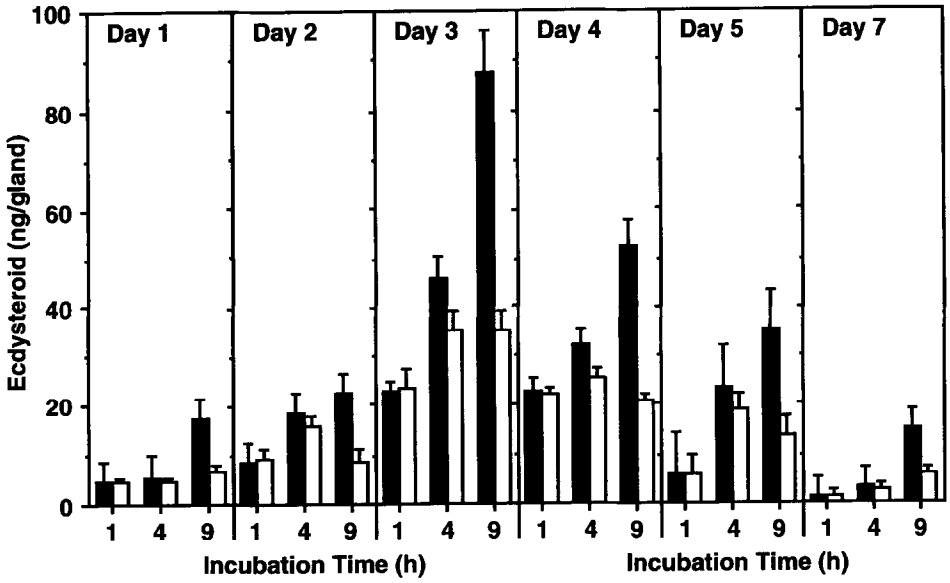

FIGURE 8. Influence of incubation time on the *in vitro* production of ecdysone (solid bars) and 3-dehydroecdysone (open bars) by prothoracic glands from different ages of female *L. dispar* pupae and pharate adults. Eclosion occurred on day 10. Glands were dissected, taken through two 5 min washes in fresh saline and incubated in Grace's medium according to protocol 2 where a different gland was used for each incubation time tested and the medium was not replaced during the incubation. Points represent means ± SE of 5 replicates of 3 gland pairs per replicate incubated for the time indicated on the *x*-axis. The media from each of the triplicate gland pairs were combined at the end of the incubation, extracted with methanol (75%) and fractionated by RP–HPLC (methanol:water, 41:59, v/v; 1 ml/min; Supelcosil LC-18-DB, 5 cm × 4.6 mm i.d., Supelco Inc., Bellefonte, PA). Known quantities of ecdysone and 3-dehydroecdysone were used as external standards to quantify the integrated peak areas.

measured at 1 and 4 h of incubation (Fig. 8). The amount of ecdysone produced at 9 h of incubation was approximately twice that of 3-dehydroecdysone ($P \leq 0.0001$). While ecdysone production continued to increase through 9 h of incubation for each age tested, 3-dehydroecdysone production peaked at 4 h for ages 2–5 days (Fig. 8). A small increase in 3-dehydroecdysone production through 9 h of incubation was observed for ages 1 and 7 days.

The data shown in Figs 1(B) and 8 were obtained using the same experimental protocol except for the method used to quantify ecdysteroid, a radioimmunoassay for Fig. 1 and reverse-phase HPLC for Fig. 8. For the 1, 4 and 9 h incubations of glands from each age tested, estimation of the amounts of ecdysteroid produced by radioimmunoassay correlated ($P > |r| \leq 0.001$, $r = 0.95$, $n = 180$) with the amounts of ecdysone estimated by reverse-phase HPLC [cf. Figs 1(B) and 8].

DISCUSSION

The amount of ecdysteroid produced by prothoracic glands *in vitro* was highest in glands from day 3 females and then declined in glands from older females [Fig. 1(A) and (B)]. These developmental differences in ecdysteroid production by prothoracic glands *in vitro* correspond temporally with the hemolymph ecdysteroid titer in female pupae and pharate adults which peaks on day 3 in females (Schnee *et al.*, 1984). Fluctuations in the hemolymph ecdysteroid titer in last instar *M. sexta* (Bollenbacher *et al.*, 1975; Ciancio *et al.*, 1986), *L. dispar* (Kelly *et al.*, 1986, 1992) and *B. mori* (Okuda *et al.*, 1985) correlate temporally with fluctuations in the *in vitro* production of ecdysone by prothoracic glands from the last instar of these species. This study is the first to report on the temporal correlation between ecdysteroid production by prothoracic glands *in vitro* and the hemolymph ecdysteroid titer in pupal and pharate adult Lepidoptera.

Ecdysteroid production *in vitro* by prothoracic glands from nondiapausing *M. sexta* pupae is influenced by 20-hydroxyecdysone (Sakurai and Williams, 1989). We tested the hypothesis that the amount of ecdysteroid produced *in vitro* could be modulated by the ecdysteroids produced by the glands. Ecdysteroids produced by the glands *in vitro* were either allowed to build up [Fig. 1(B)] or were periodically removed and replaced with fresh medium [Fig. 1(A)]. Within each pupal and pharate adult age from which glands were obtained, the amount of ecdysteroid produced *in vitro* was similar for each of the incubation protocols [Fig. 1(A) and (B)]. The two ecdysteroids (ecdysone and 3-dehydroecdysone) observed to be produced by the prothoracic glands from female *L. dispar* pupae and pharate adults may not be involved in feedback regulation of ecdysteroid production in pupae and pharate adults.

The stimulation of the amount of ecdysteroid produced *in vitro* by brain extract was greatest with prothoracic glands from day 2 and 3 female pupae and pharate adults, and then declined in glands from older females (Fig. 2). Sensitivity of ecdysteroid production *in vitro* to the dose of brain extract was highest (lowest ED_{50}) in glands from day 1–3 females and lowest in glands from older females

(Fig. 6). Glands from day 4 and older females were almost completely refractory to stimulation by brain extract. Prothoracic glands from female *L. dispar* pupae and pharate adults appear to be most competent to respond to a PTTH stimulus on day 2 after the larval–pupal molt. Developmental fluctuations in both the ability of glands to produce ecdysteroid *in vitro*, and the competency of glands to respond to a PTTH stimulus *in vitro*, correspond temporally with the *in vivo* developmental fluctuations of ecdysteroids (e.g. 20-hydroxyecdysone) in the hemolymph which peak on day 3 in females (Schnee *et al.*, 1984). Internal pharate adult features, such as antennae, optic lobes of the brain and breakup and histolysis of the fat body, begin to develop 3 days after the larval–pupal molt. Also occurring at this time are patency and the onset of vitellogenin uptake by the ovaries (Davis *et al.*, 1990; Lamison *et al.*, 1991). Thus, apolysis of the pupal cuticle and the initiation of oogenesis and pharate adult development are probably stimulated by the increase in the hemolymph ecdysteroid titer during the first 3 days after the larval–pupal molt. This increase in the hemolymph ecdysteroid titer appears to depend not only on the amount of ecdysteroid produced by prothoracic glands modulated by PTTH, but, in addition, some factor which determines the competency of glands to respond to PTTH. *L. dispar* pupae and pharate adults are very different from day 5–10 last instar female larvae whose competency of glands to respond to PTTH declines with increases in the ability of these glands to produce ecdysteroid *in vitro* and the hemolymph ecdysteroid titer (Kelly *et al.*, 1992). The gland competency during the first 3 days after the larval–pupal molt may be regulated by the hormonal cascade associated with post-pupal commitment in the last instar and the larval–pupal molt; i.e. decline in the second peak in the juvenile hormone titer of larvae (Bollenbacher, 1988) or fluctuations in the titers of eclosion hormone, bursicon or both (Reynolds, 1986). The release of PTTH in pupae has been shown to occur in *M. sexta* (Bell *et al.*, 1975; Bowen *et al.*, 1984). Since gland competency during the first 3 days after the larval–pupal molt of *L. dispar* coincides with the ability of glands to produce ecdysteroid *in vitro*, it is also conceivable that the release of PTTH just after this molt may contribute to gland competency in pupae and pharate adults as it may in day 2 *M. sexta* larvae (Wolfgang and Riddiford, 1986; Bollenbacher, 1988).

Decrease in both the ability of glands to produce ecdysteroid *in vitro* and gland competency after day 3 is probably related to the degeneration of the prothoracic gland which commonly occurs in pharate adults of *L. dispar* (H.W.F., unpublished observation) or other Lepidoptera (Herman and Gilbert, 1966; Blazsek *et al.*, 1975; Sedlack, 1985). This is the first study to report that there is a relationship in pupal and pharate adult Lepidoptera between gland degeneration and the decrease in ecdysteroid production by glands *in vitro*, gland competency and the hemolymph ecdysteroid titer. The hemolymph ecdysteroid titer rapidly decreases between days 3 and 5 to about 85% of the peak on day 3 (Schnee *et al.*, 1984). Degeneration of the prothoracic glands is first evident 4 days after the larval–pupal molt when glands first lose their translucence and become an opaque white color. Glands then progressively shrink in size until they are no longer present in pharate adults 10 days after the larval–pupal molt. Adults emerged during the early photophase of the next day (H.W.F., unpublished observation; Schnee *et al.*, 1984). Like other Lepidoptera (Herman and Gilbert, 1966; Sedlack, 1985), *L. dispar* adults do not have prothoracic glands (H.W.F., unpublished observation). Perhaps gland degeneration is being influenced by the ecdysteroid titer in pupal and pharate adult hemolymph which could also be modulating gland competency by direct stimulation or inhibition of ecdysteroid production. Such an effect was reported for 20-hydroxyecdysone in *M. sexta* larvae and pupae (Sakurai and Williams, 1989).

The amount of ecdysteroid produced by prothoracic glands *in vitro* was constant after the first hour of incubation [Fig. 1(A) and (B)] indicating that a 1 h pre-incubation with no stimulus in the medium would be a standard part of an *in vitro* bioassay for PTTH activity using glands from female *L. dispar* pupae and pharate adults. Kinetic [Figs 1(A), (B) and 2] and dose–response (Fig. 6) studies of glands from different pupal and pharate adult ages indicate that glands from day 2 females have the greatest response to brain extract. Incubation of these glands with brain extract (Fig. 2) results in enhanced, linear increase in ecdysteroid production for the first 3 h following stimulation. Therefore, glands from day 2 female *L. dispar* pupae or pharate adults were used in an *in vitro* bioassay for detection of PTTH activity. The standard bioassay developed to quantify PTTH activity consists of the gland pairs incubated without stimulus for 1 h followed by a 3 h incubation of the experimental gland with stimulus, such as a particular dose (brain equivalent/25 μl) of a boiled extract of brains from day 2 females. This procedure resulted in the greatest activation ratio (see above). Similar *in vitro* bioassays for the quantification of PTTH activity have been developed with prothoracic glands from *L. dispar* larvae (Kelly *et al.*, 1986, 1992), *B. mori* larvae (Agui *et al.*, 1983; Okuda *et al.*, 1985) and *M. sexta* larvae and pupae (Bollenbacher *et al.*, 1979, 1983).

PTTH activity in brain extracts was not significantly influenced by boiling [Fig. 3(A) and (B)] indicating that the extract contained a heat stable PTTH factor. Similar findings were reported for *L. dispar* larvae (Masler *et al.*, 1986) and other Lepidoptera (Ishizaki *et al.*, 1983; Bollenbacher *et al.*, 1984). Differences between the dose–responses of prothoracic glands from day 2 female pupae to extracts of brains from day 1 or 2 female pupae or pharate adults (Fig. 5) indicates that the relative amount of PTTH activity in pupal brains varies with development. Similar findings were reported for *M. sexta* larvae and pupae (O'Brien *et al.*, 1986). Only the brain and corpora allata of female *L. dispar* pupae or pharate adults (Fig. 4) possessed PTTH activity, as was also

reported for *M. sexta* larvae (Agui *et al.*, 1980). Like *M. sexta* larvae (Agui *et al.*, 1980; Tomioka and Bollenbacher, 1989), the corpus allatum is probably the neurohemal organ for the prothoracicotropes in *L. dispar* pupae and pharate adults.

There is some confusion and disparity in the literature as to what kind of biological activity to use when designating a particular molecule as a PTTH. For example, bombyxin (previously termed 4K-PTTH; Ishizaki and Suzuki, 1984) from *B. mori* is a 4.4 kDa insulin-like peptide (Nagasawa *et al.*, 1984b, Nagasawa *et al.*, 1986) which is no longer considered by some to be a PTTH (Bollenbacher *et al.*, 1993) because it has no *in vivo* activity in *B. mori* (Suzuki and Ishizaki, 1986). However, bombyxin has *in vivo* and *in vitro* activity in *Samia cynthia ricini* (Drury) (Lepidoptera: Saturniidae) (Ishizaki and Ichikawa, 1967; Nagasawa *et al.*, 1984a) and it stimulates *in vitro* production of ecdysteroids by *B. mori* (Fujimoto *et al.*, 1991) and *S. cynthia ricini* prothoracic glands (Nagasawa *et al.*, 1984a). The term PTTH will be used for the purpose of this discussion to indicate a molecule fractionated by molecular sieving that stimulates *in vitro* production of ecdysteroids by prothoracic glands. Extracts of brains and corpora allata fractionated by high performance size exclusion chromatography with an acidic organic solvent (Fig. 7) showed PTTH-activity peaks at 3.2 and 13.75 kDa, designated by Masler *et al.* (1986) for female *L. dispar* larvae as small (PTTH-II) and big (PTTH-I), respectively. The presence of at least two molecular sizes with PTTH-activity in female *L. dispar* larvae (Masler *et al.*, 1986; Kelly *et al.*, 1992), pupae and pharate adults is consistent with the presence of small and big molecular weight peptides with PTTH-activity in all species reported thus far in the literature. These species include *B. mori* adults (Ishizaki *et al.*, 1983; Ishizaki and Suzuki, 1984; Fujimoto *et al.*, 1991), *M. sexta* larvae (Bollenbacher *et al.*, 1984, 1993) and pupae (O'Brien *et al.*, 1986; Bollenbacher *et al.*, 1993) and the larvae of other species of Lepidoptera (Fujimoto *et al.*, 1991). The 3.2 kDa PTTH-active molecule in the brain and corpora allata of female *L. dispar* pupae and pharate adults is smaller than the small PTTH-active molecules reported for *L. dispar* larvae (about 5 kDa; Masler *et al.*, 1986; Kelly *et al.*, 1992), *B. mori* (about 4 kDa; Nagasawa *et al.*, 1984a; Ishizaki and Suzuki, 1984) and *M. sexta* (about 7 kDa; Bollenbacher *et al.*, 1984, 1993; O'Brien *et al.*, 1986). The 13.75 kDa PTTH-active molecule in the brain and corpora allata of female *L. dispar* pupae and pharate adults falls within the approximately 12.5 and 15 kDa molecular sizes found in the brain of female larvae by Kelly *et al.* (1992) and Masler *et al.* (1986), respectively. Kelly *et al.* (1992) used extraction and fractionation conditions identical to those of this study. All of these molecular sizes for the big PTTH-active molecule in *L. dispar* are smaller than those reported for large PTTH in *B. mori* (about 22 kDa; Ishizaki *et al.*, 1983; Ishizaki and Suzuki, 1984) and *M. sexta* (about 28 kDa; Bollenbacher *et al.*, 1984, 1993; O'Brien *et al.*, 1986). There is no overlap between the small molecular weight range of 3–7 kDa and the big molecular weight range of 13–30 kDa when all species with PTTH-active molecules are considered. The reason for the variation between species is not clear at present. This variation could be due to differences in the degree of glycosylation or the conditions used for fractionation; i.e. denaturing versus non-denaturing conditions. The degree of homology between the PTTHs in each species will be resolved only when the complete chemical structures are known for each PTTH.

Glands from all species and stages of Lepidoptera that have been examined, including *L. dispar* larvae (Kelly *et al.*, 1990), produce ecdysone and 3-dehydroecdysone *in vitro* (Kiriishi *et al.*, 1990). Prothoracic glands from each age of *L. dispar* pupae and pharate adults tested in the current study also appear to produce ecdysone and 3-dehydroecdysone *in vitro*. The ratio of ecdysone to 3-dehydroecdysone produced by the prothoracic glands *in vitro* varies widely among the Lepidoptera (Kiriishi *et al.*, 1990). Glands from day 1 *M. sexta* pupae produce an ecdysone to 3-dehydroecdysone ratio of 1:7 (Warren *et al.*, 1988; Kiriishi *et al.*, 1990) while glands from wandering larvae of *B. mori* produce a ratio of 1:0.03 (Kiriishi *et al.*, 1990). Glands from *L. dispar* pupae and pharate adults initially (1–4 h) produced similar amounts (approximate ratios of 1:0.9 for 1 h and 1:0.8 for 4 h) of ecdysone and 3-dehydroecdysone *in vitro*, but the production of 3-dehydroecdysone peaked after 4 h of incubation and began to decrease (Fig. 8). In contrast, the production of ecdysone continued to increase with incubation time (approximate ratio of 1:0.4 for 9 h). Thus, the ecdysone to 3-dehydroecdysone ratio increases during incubation.

The 3-dehydroecdysone produced by *M. sexta* glands is converted to ecdysone by a 3-oxoecdysteroid 3β-reductase (3β-reductase) in the hemolymph (Warren *et al.*, 1988; Sakurai *et al.*, 1989b). Various methods designed to remove reductase activity from prothoracic glands, including thorough rinsing of the gland with saline, were tested by Kiriishi *et al.* (1990). Failure of these methods to alter the ratio of ecdysone to 3-dehydroecdysone produced by *M. sexta* glands suggested that both ecdysteroids are *de novo* products of cells comprising the prothoracic glands. Although the glands used in our study were rinsed well with saline before incubations were begun, it is possible that conversion of 3-dehydroecdysone to ecdysone by residual 3β-reductase in hemolymph clinging to the glands could be responsible for the decrease in 3-dehydroecdysone and concomitant increase in ecdysone after 4 h of incubation. The different amounts of ecdysone and 3-dehydroecdysone produced in female *L. dispar* pupal and pharate adult glands may also reflect differences in *de novo* synthesis. Thus, the contribution of both synthesis and conversion need further explanation.

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Acknowledgements—We are grateful for the assistance of R. A. Bell in rearing the insects. We thank N. Le, C. Masler, C. Robinson and D. Vu for technical assistance. Mention of a commercial product does not constitute endorsement of that product by the USDA. This paper is technical contribution No. 3477 of the South Carolina Agricultural Experiment Station, Clemson University. H. W. Fescemyer was supported by USDA Competitive Grant No. 86-CRCR-1-2111 to E. P. Masler and T. J. Kelly.